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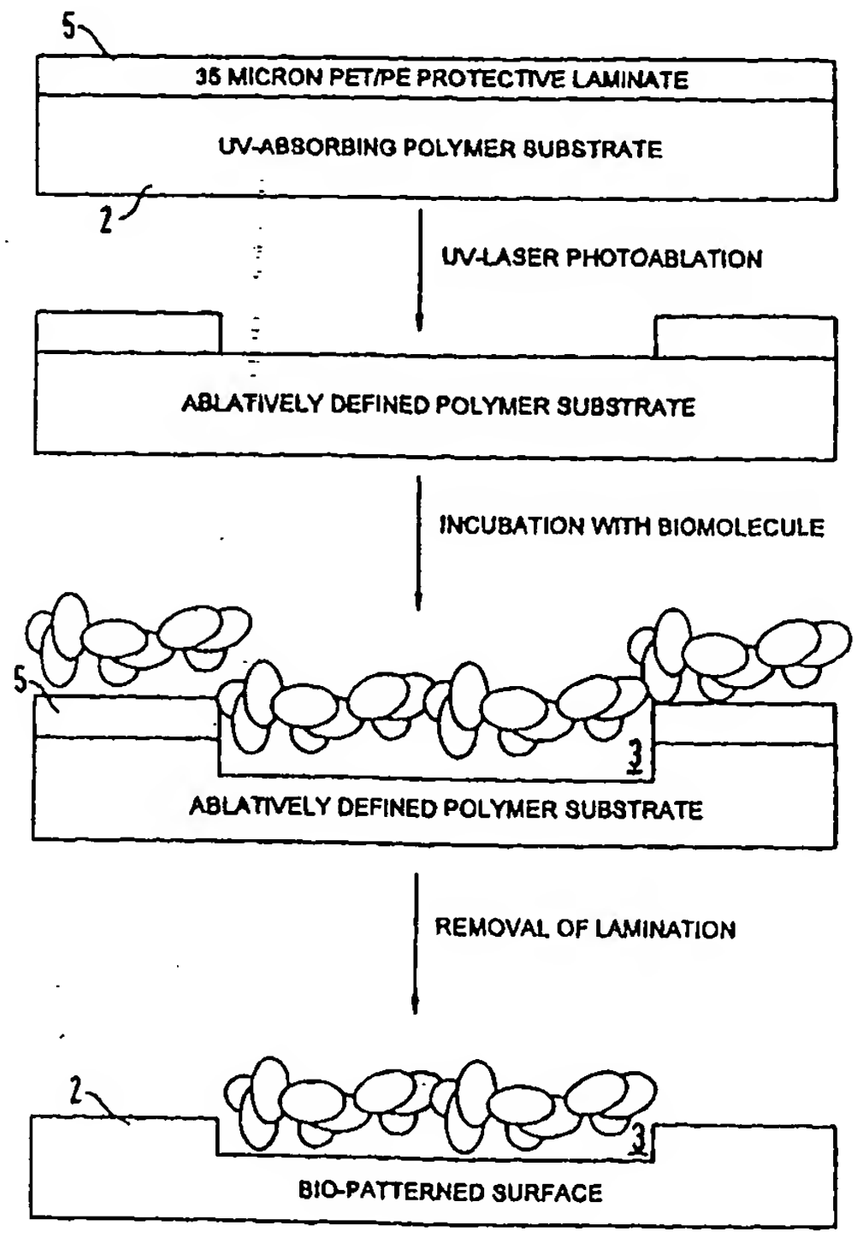
(54) Title: SURFACE PATTERNING OF AFFINITY REAGENTS USING PHOTOABLATION

(57) Abstract

UV–laser photoablation is used for the 3–dimensional patterning of biological and chemical substances onto polymer and other UV–absorbing substrates. This method creates ablated lines, holes, or entire networks of structures which may selectively contain a chemical substance of interest and have critical dimensions in the range of 1 – 1000 μm. High–energy pulses are fired at a protected polymer substrate (2) from an UV excimer laser, thereby creating an ablated cavity (3) which passes through the protective layer and into the underlying substrate. Complex geometrical structures may be fabricated by repetitive firing of the laser through a series of mask onto stationary substrates. The resulting ablated–polymer structures show increased rugosity which enhances the surface area for binding chemical or biological receptors, including enzymes, antibodies, nucleic acids, other polymers, gels, membranes, etc. Binding may then be accomplished via simple adsorption or through covalent and/or noncovalent conjugation to the entire surface (both ablated and non–ablated). After the binding step, the protective layer (5) can simply be peeled off, thereby removing the binding material from all surfaces, except that which is defined by UV–laser photoablation. The resulting surface is then left in a state which is chemically and geometrically defined by the initial UV–laser exposure.



SURFACE PATTERNING OF AFFINITY REAGENTS USING PHOTOABLATION

Background to the Invention

Many recent advances in chemical analysis have involved the incorporation of biomolecules capable of selective and high affinity binding to analytes of interest. Such devices are often termed biosensors, which involve real-time transduction of the binding event into an electronic signal, but also include analytical technology such as, for example, affinity chromatography, immunoassay and enzyme reaction, as well as nucleic acid hybridization, which can be performed in analytical polymerase chain reaction (PCR) devices. Bio-analytical devices utilizing this technology have been applied to a wide range of applications in medicine, agriculture, industrial hygiene, and environmental protection. There is also growing interest in the use of modified materials in bio-medical implants, prosthetic devices, catheters, etc. Furthermore, there is an emerging field of bio-electronic and bio-mimetic materials, which can be used for information processing, that rely on the transduction of some molecular recognition event[1].

Recently, there has also been intense activity in the miniaturization of chemical instrumentation. Efforts have been made to reduce whole laboratory systems on to microchip substrates often utilizing capillary electrophoresis (CE) as the principal analytical technique[2]. These systems have been termed micro-Total Analytical Systems (μ-TAS)[3]. Some promising bioanalytical applications have already been demonstrated which are based on immobilized receptors within microfabricated fluid handling platforms[4-7].

Immobilization technology is critical to the further development of all of these technologies. In many cases, commercialization of new ideas has been hampered by either inadequate, unstable, or unreproducible immobilization of the biomolecule components[8]. Immobilization has been accomplished in the past by adsorption, entrapment, non-

that the disclosed technique of Ligler et al is clearly not UV-photoablation but only UV-irradiation. UV-photoablation requires significantly higher laser pulse energies then were used in that study and is a completely distinct physical phenomenon, which the present inventors use to advantage in the bio-patterning of biomolecules and chemical substances.

Summary of the Invention

Accordingly, it is one object of the invention to provide a novel method for the patterning of biomolecules and chemical substances in 2 and 3-dimensional patterns on UV-absorbing substrate materials.

It is another object of the present invention to provide material surfaces with enhanced immobilization capacity for biomolecules such as proteins, nucleic acids, and lipid films by physical adsorption.

It is another object of the present invention to provide material surfaces with enhanced functionalization of chemical groups for subsequent covalent conjugation procedures.

It is another object of the invention to provide surfaces with protective layers such as adsorbed non-specific protein layers, chemically conjugated lipid films, laminated polymers, adhesives, or photoresists which can be patterned using UV-laser photoablation.

It is yet another object of the invention to utilize the UV-laser ablated, protected surfaces as a contact mask for the subsequent patterning of biomolecules and chemical substances onto the underlying polymer substrate.

These and other objects, which will become apparent during the following detailed description of the present invention, have been achieved by the inventors' use of UV-laser photoablation (as opposed to simple irradiation),

patterning using UV-laser photoablation;

Figure 4b shows a control in which no avidin was used;

Figure 5 is a voltammogram demonstrating β-Galactosidase bio-patterning using UV-laser photoablation;

Figures 6a to 6c are schematic diagrams of a UV-laser defined microchannel for concentration and separation of analytes in solution;

Figure 7 is a scanning electron micrograph (SEM) of a UV-laser fabricated micro-well array for nucleic acid analysis; and

Figure 8 shows a sandwich immunoassay facilitated by UV-laser patterning.

Detailed Description of the Preferred Embodiments

Machining and characterization of UV-laser photoablated micro-patterns.

Structure may be formed in various commercially available polymers by photoablation upon interaction with UV laser radiation ($\lambda < 330nm$; fluence > 0.5 J cm^{-2} per pulse). These have been reviewed[14], but include cellulose acetate, nitrocellulose, polycarbonate, polyimide, poly(methyl methacrylate) (PMMA), polystyrene, poly(ethylene terephthalate) (PET), and poly(tetrafluoroethylene). The photoablation process involves absorption in the UV region with concomitant electronic transitions from the ground singlet state to the first excited singlet states, rapid bond breaking within the long chain polymer molecules, and then ejection of substrate, in a mini-explosion, from the surface leaving a photoablated cavity[14] 3, as depicted in Figure 1b.

The laser energy can be specifically patterned using a photomask with the subsequent generation of microcavities and channels of various geometry. The laser energy may also be patterned in even smaller dimensions by using fiber-optic light guides. Such light guides are capable of handling UV-laser energy of several hundred mJ per pulse and can be fashioned with small tips, using techniques known to those

bovine serum albumin, chicken ovalbumin, human serum albumin, any other albumin, non-fat dry milk (NFDM) or any other suitable protein solution capable of efficiently and homogeneously coating the substrate material. An example of an adsorbed protein protective layer 4 is shown in Figure 2a. This protective layer may also be composed of chemically conjugated species such as lipid monolayers, self-assembled layers, fatty acid chains, carbohydrate moieties, or other suitable substances. An alternative protective layer is composed of a polymer laminate such as poly(ethylene terephthalate)/polyethylene (PET/PE) for example, or any other suitable polymer possessing the characteristics of low-temperature and rapid adhesion to the base substrate. An example of a laminated protective layer 5, adherent to a polymer substrate, is shown in Figure 3a. This protective layer may also be composed of other types of materials applied by various techniques which include but are not limited to adhesives, screen printed polymers, and spin-coated photoresist layers. It should be clear to those skilled in the art that the substances mentioned here are a small subset of the possible protective layers that may be applied to UV-laser photoablative substrates, and that these layers should only serve to create a general mask for the substance to be patterned. It should also be realized that such a mask can be both positive or negative, in that the chemical properties of the mask can either block or attract the species of interest in some semi-permanent or complete manner. The subsequent UV-ablative step will serve to define the protected surface into distinct areas possessing this chemical property (non-ablated) and areas that do not (ablated).

To obtain an adsorbed protective layer as shown in Figure 2a, substrate samples can simply be immersed in a high concentration of BSA or NFDM (10 mg/mL in 60 mM phosphate buffered saline (PBS), pH 7.0) at room temperature for 2 - 4 hours. Such solutions of "nonspecific" proteins are known to bind in high concentrations to polymer materials, which in their unablated form have been used for

An affinity reagent was allowed to physically adsorb to the photoablated polymer through laser defined openings in a protective layer. In the case of a PET/PE protective laminate, the lamination is simply peeled off after thorough washing, leaving only laser-defined areas of immobilized affinity reagent as shown in Figure 3d.

It should also be understood by those skilled in the art that more complex geometries can alternatively be produced by utilizing more complicated photomasks. Using contact lithography or other techniques, which are well known in the integrated circuit manufacturing industry, it is possible to create complex ablated patterns. The photomasks are simply placed in front of the excimer laser and repeated pulsing will ablatively pattern the substrate through differnt masks.

Characterization of Photoablated Cavities

The relationship between the number of laser pulses, laser frequency, and the resulting μ-channel depth has been studied by SEM[13]. Samples were generated using a static substrate and a photomask of the dimensions previously described. High energy UV-laser pulses (2 J cm^{-2} per pulse) were focused on a polycarbonate surface and 0 - 1000 pulses were fired at 10, 20, and 50 Hz. Samples were then analyzed by scanning electron microscopy (SEM). The channel depth could be determined in this manner using a high tilt angle (60°) and subsequent measurements of an exposed depth profile. The channel depth was found to be essentially linear (and did not vary with frequency) according to the relationship: Depth (μm) = 0.17*N, where N = the number of laser pulses.

In many cases surface patterning of chemical substances will be the desired result and, therefore, only a low number of pulses will be required to pattern the protective layer, thereby creating little actual structure in the substrate. However, in more complicated devices, especially with fluid

terephthalate) showed the largest increase with an overall depth variation of 0.40 μm. From the perspective of developing microfabricated diagnostic assays, the increased surface area seen particularly with photoablated poly(ethylene terephthalate) could be very useful for the deposition of receptor and ligand zones. The magnitude of chemical adsorption will be related to the photoablatively induced charge and the surface area of the resultant microstructure. The increase in surface area due to the observed increase in rugosity leads to increased adsorption.

The adsorptive capacity of photoablated polymers was also studied, using BSA as a model protein intended to show relative differences among the various surfaces studied and the results can be seen in Table 2.

Table 2

Polymer	BSA on Ablated μg/cm²	BSA on Non Ablated μg/cm²	Adsorption factor
Collulose Acetate	6.7	0.5	13.2± 7.3
PET	91.3	1.1	82.3±15.5
Polystyrene	145.1	2.8	52.0± 4.0
Polycarbonate	174.3	4.3	40.4± 5.1
Polyimide	241.1	3.7	65.1± 9.5

Unablated polymer substrates showed little difference in protein adsorption with an average of 1.32 μg BSA / cm² as measured by the Bicinchoninic Acid (BCA) test (Measures reduction of copper by amino acids[14]). This value is higher than the 0.4 μg protein / cm² reported in the literature[9]. Our observations were only for BSA, a protein which can be expected to show differences in adsorption when compared to a generalized observation of several different proteins.

Although the BCA test measures the protein's ability to reduce copper, which may be enhanced by any denaturation caused by adsorptive immobilization, the test can be expected to accurately determine relative differences in the various surfaces examined here.

be 2-dimensionally patterned by defining the UV-laser beam geometry. All of the BCA tests on photoablated polymers were done over 1 mm diameter ablated wells and after removal of the laminate, no protein could be measured outside of the ablated area. Therefore the 35 μm thick PET/PE laminated protective layer has been observed to completely block non-specific protein binding to areas outside of the laser ablated regions. This is a significant advantage over the prior art in bio-patterning.

It is also demonstrated here that a model receptor, avidin from egg whites, can be immobilized in such microfabricated patterns. This experiment will demonstrate two additional concepts central to bio-patterning using the technique of UV-laser photoablation; 1) Receptors can be immobilized onto photoablated surfaces while maintaining binding activity for a ligand and 2) that receptors bound in this manner can be patterned with high resolution.

Avidin was allowed to physically adsorb to photoablated polycarbonate through laser defined openings (1 mm diameter microwells) in a PET/PE protective laminate as previously described and shown in Figure 3c. After washing, the lamination was peeled off leaving only laser-defined areas of immobilized avidin as shown in Figure 3d. Biotinylated liposomes have been previously shown to have high affinity for avidin in solution as well as avidin immobilized to polymer surfaces[12, 15-17]. Ten μL solutions of liposomes, containing 0.1 mol% dipalmitoyl phosphatidyl choline-biotin conjugate (Molecular Probes, Eugene Oregon) and 200 mM encapsulated sulpharhodamine-B, were incubated for 2 hours at room temperature over the photoablated microwells and control surfaces. The entire surface of the polycarbonate sample was then vigorously washed with 67 mM PBS for 60 seconds. Liposomes remained attached to the surface of UV-laser defined microwells through an avidin-biotin non-covalent linkage, as demonstrated in Figure 4a. It was also shown that there was little or no liposome binding to laser-treated areas which were not incubated with avidin, as seen

It should also be noted that the covalent attachment of affinity matrices may also be defined using UV-laser photoablation for purposes similar to those described above. Because the photoablative process is observed to produce chemically modified surfaces the photoablated, non-blocked section of polymer being defined is also available for covalent modification. Simple peptide linkages may be formed between these surface functional groups and amino acid side chains existing on protein structures such as enzymes and receptors. This may be accomplished by using commercially available reagents, such as Dicyclohexlcarbodiimide and N-Hydroxysuccinimide. Proteins, phospholipids, amino acids, and other components of interest may be attached in this manner or with other suitable methods of chemical attachment. It should be emphasized that if the initial blocking step is sufficient then any manner of these attachments will be localized to the region specified by the UV-laser machining process.

In an alternative embodiment of the invention, the initial unablated polymer substrate is completely blocked by first applying a laminate. In this embodiment, the laminate is a 35 μm PET/PE layer and is annealed to the base polymer at 125 °C for 2-3 seconds. The laser is used to etch a defined pattern into the laminate by firing repeated pulses sufficient to eventually pierce the laminate and create structures of desired depth into the base polymer. The resulting patterned laminate-substrate structures can then be easily immersed into solutions of affinity reagents. As an example, a protein receptor solution, composed of IgG antibodies dissolved in 200mM borate buffer (pH 9.0), may be incubated at room temperature for approximately two hours over the aforementioned structures. The structure is then washed with clean buffer, containing no antibodies, and the laminate is peeled off, as shown in Figure 3. All antibodies bound to the laminate are removed with the lamination, and the only remaining regions with adsorbed receptor are those defined by the UV-laser machining process. A small number of laser pulses can then be used on areas of the substrate where antibodies are not desired, thereby providing a cleaning step. This preferred method may, in some cases, be advantageous over the protein-blocking method, which is sufficient for many analytical applications but does not block 100 % of the reagent. In contrast, removal of reagents bound to the laminate by peeling leaves no possibility for adsorption in nonablated regions.

One possible embodiment of the invention utilizing a UV-laser defined reactive area within a microfabricated fluid handling system is shown in Figure 6. Here a biological affinity reagent 60, which may be any protein receptor (i.e., antibodies, enzymes, etc.), ligand, or nucleic acid (DNA, RNA, tRNA, etc.) can be defined in an area as small as 5 x 5 μm within a photoablated capillary 61. As an example, but by no means as a limiting case, an anti-polychlorinated biphenyl (anti-PCB) antibody may be deposited in such a zone. A sample containing several PCB congeners could then be introduced and continually pumped

For the present example, a preferred substrate will be polystyrene which is coated with a 35 μm thick PET/PE laminate (applied with pressure at 125 °C for 3 seconds). Polystyrene has been previously shown to physically adsorb DNA, and furthermore, in a fashion that allows subsequent hybridization[11]. Photoablated polystyrene has now been shown to be advantageous for the immobilization of biomolecules and therefore provides a surface with greater immobilization capacity for nucleic acid probes. Therefore, adsorption will be used as a convenient immobilization procedure for nucleic acid probes used in this example. After lamination, small holes of approximately 10 μm in diameter are ablated through the protective layer with 200 pulses of 1.6 J cm^{-2} per pulse. UV-laser photoablation is continued into the polystyrene substrate with a further 60 pulses creating 10 μm holes of approximately 10 μm in depth.

Solutions of nucleic acid sequences, prepared as known to those skilled in the art, which can be used as probes, are dissolved in high salt buffers (500 mM NaCl) and placed in small droplets (10 μL) over UV-laser ablated microwells for 2-4 hours at room temperature. After this initial incubation period the patterned microwell(s) is washed with buffer to remove any excess probe and then air dried. Using a step and repeat process different primary probe solutions are added to the growing array. One possible embodiment is shown in Figure 7, where a completed 2-dimensional array of microholes is shown with 100 μm spacing. Finally, the PET/PE protective laminate is peeled off removing any probe not immobilized in the microwell.

The device now consists of multiple photoablated wells 2-dimensionally defined with numerous different nucleic acid probes, capable of hybridization with sequences of medicinal and toxicological interest in a test sample. Subsequently, an unknown solution is placed in bulk over the entire array. Each of the immobilized probes will then specifically hybridize with a complementary sequence, if present, in the original test solution. A second set of probes, containing

polystyrene substrate with a further 200 pulses creating 100 μm holes of approximately 50 μm in depth. One possible embodiment is similar to that shown in Figure 7, but with a 10 x 10 microhole array, which has holes spaced 3 mm apart in either the vertical or horizontal dimensions. The larger dimensions described in the present example are for separation and easier addressability of the numerous test solutions to be applied. It should be noted though, that the entire "foot print" of such a test plate is only 3 x 3 cm whereas, a conventional 96-well microtiter plate is approximately 8.5 x 12.5 cm.

In a preferred sandwich immunoassay embodiment of the present example, a 500 μg / mL solution of avidin, from chicken egg whites, in PBS is allowed to incubate over the entire array for two hours at room temperature. The technique of pre-adsorbing avidin prior to application of a specific antibody solution allows the immobilization of any biotinylated antibody, receptor, or marker reagent. Furthermore, this technique will preserve more of the receptor's original activity by preventing close interaction with the substrate which leads to a certain amount of denaturation. If a biotinylated antibody reagent is unavailable or inconvenient to synthesize then the antibody can also be directly immobilized to the surface using the method of the present invention. In this case, after the avidin solution has adsorbed to the surface then the array plate is thoroughly washed with PBS and dried with pressurized air before application of the specific antibody solution.

In the present example, an antibody solution consists of anti-αFP in buffer (500 μg / mL in PBS), which is used for subsequent monitoring of neonatal health. It should be realized that this is a non-limiting case and that any suitable antibody can be substituted for anti-αFP, depending upon the desired analytical measurement. The solution of biotinylated anti-analyte antibody is allowed to incubate over the entire array for two hours at room temperature,

solutions. After incubation for 30 minutes at room temperature a second 2 μL aliquot of a β-Gal substrate solution (1 mM APG in 0.2 M phosphate buffer, pH 7.4 with 1 mg / mL $MgCl_2$) is allowed to react with the enzyme conjugate for a period of time that will be determined by the sensitivity desired of the assay. If the greatest sensitivity is desired than this time could be as long as 4 hours, however, if assay speed is the critical component than this time could be as short as 30 minutes.

A two electrode microprobe (working electrode = screen printed carbon, reference = screen printed Ag/AgCl paste), or any other suitable 2 electrode system capable of measuring 2-4 μL solutions, is then inserted into substrate solutions over the microwells. The potential of the working electrode is poised at +200 mV and the current is measured using a suitable commercially available current monitoring apparatus. The amount of current should be directly proportional to the amount of anti-αFP antibody-β-Gal conjugate that was bound to the microwells. The amount of conjugate is in turn directly proportional to the amount of αFP analyte that was bound to the UV-laser defined anti-αFP antibody primary coating. The final molecular configuration for detection of αFP is shown in Figure 8b.

Therefore, this example defines one possible use for the present invention of bio-patterning for the creation of microwell assay plates capable of detection of proteins of interest using the technique of sandwich immunoassay. The present method of patterning anti-analyte antibody is advantageous in that the diffusional environment for all reaction steps is reduced, the use of small volumes for samples and expensive reagents is facilitated, and the overall "foot print" of the assay plate is considerably reduced. The present method of patterning is also particularly advantageous in that there will be a large increase in the amount of primary antibody bound to the substrate relative to conventional polymer substrates. This will further increase the sensitivity of the assay as well

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patterned by UV-laser photoablation, creating one or more openings in a 2-dimensional pattern with micron resolution.

9. A method according to claim 8, wherein a solution of the affinity reagent is attached to the underlying UV-absorbing material via the UV-laser defined opening(s) through the masking layer.

10. A method according to claim 9, wherein said other material is a protective polymer laminate, which is removed after attachment of the affinity reagent to the UV-absorbing material.

11. A method according to claim 9 or 10, wherein the method of attachment is via physical adsorption, non-covalent binding, or covalent chemical conjugation.

12. A method according to claim 11, when dependent upon claim 2, wherein the biomolecule is a protein receptor, a carbohydrate conjugated protein, an enzyme, or a nucleic acid.

13. A method according to any preceding claim, wherein patterning occurs in 3 dimensions by using the UV-laser photoablative process to create structural depth upon which biomolecules may be subsequently be attached.

14. A biological or chemical sensor comprising a substrate of UV-absorbing material, carrying an affinity reagent in a specific pattern, the reagent being attached to at least one chemically functionalized and/or rugged region of the substrate.

15. A sensor as claimed in claim 14, wherein the UV-absorbing material comprises a polymer.

16. A sensor as claimed in claim 15, wherein the polymer is selected from cellulose acetate, polystyrene, polycarbonate, poly(ethylene terephthalate) and polymide.

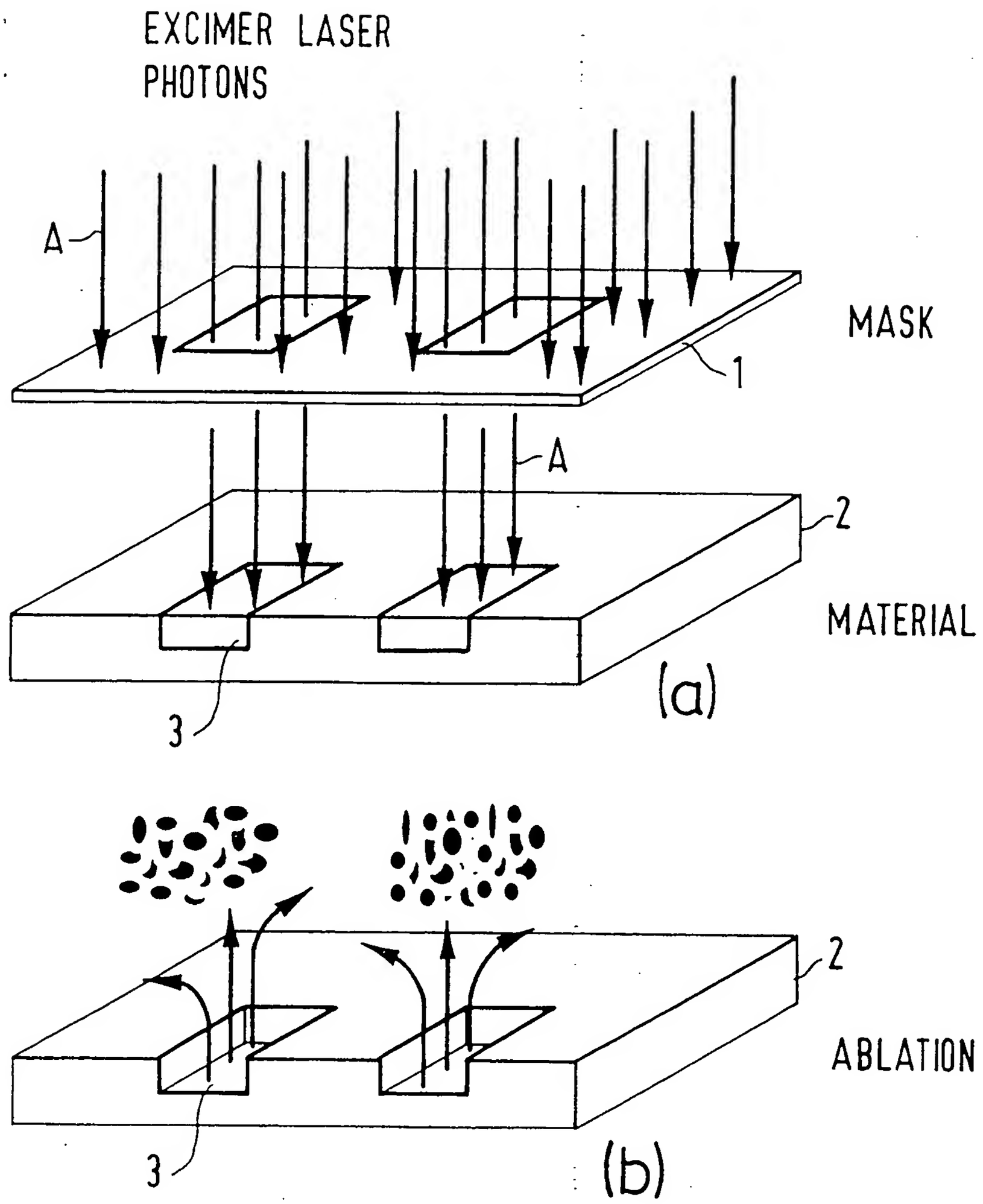

FIG. 1

FIG.3

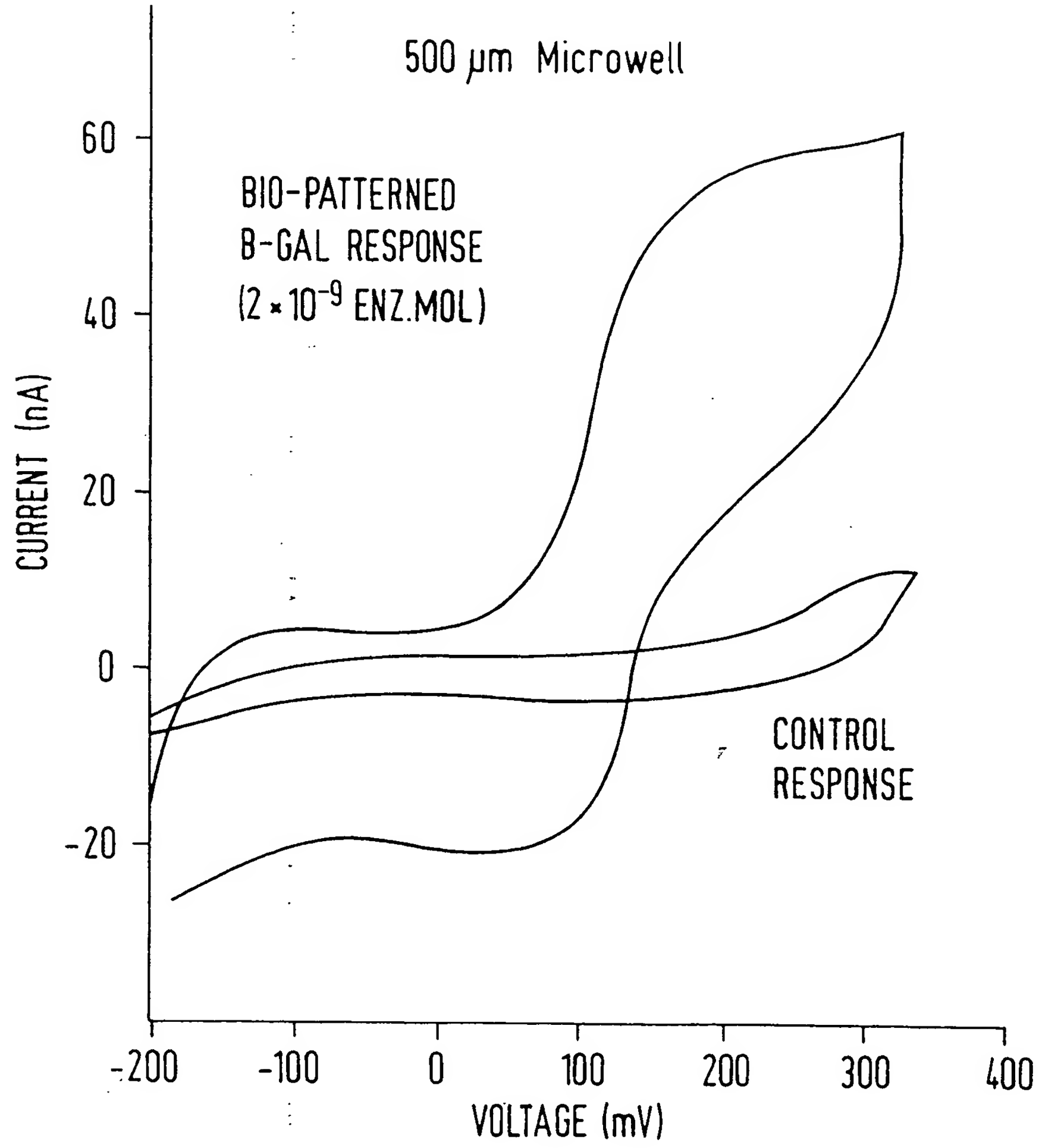

FIG. 5

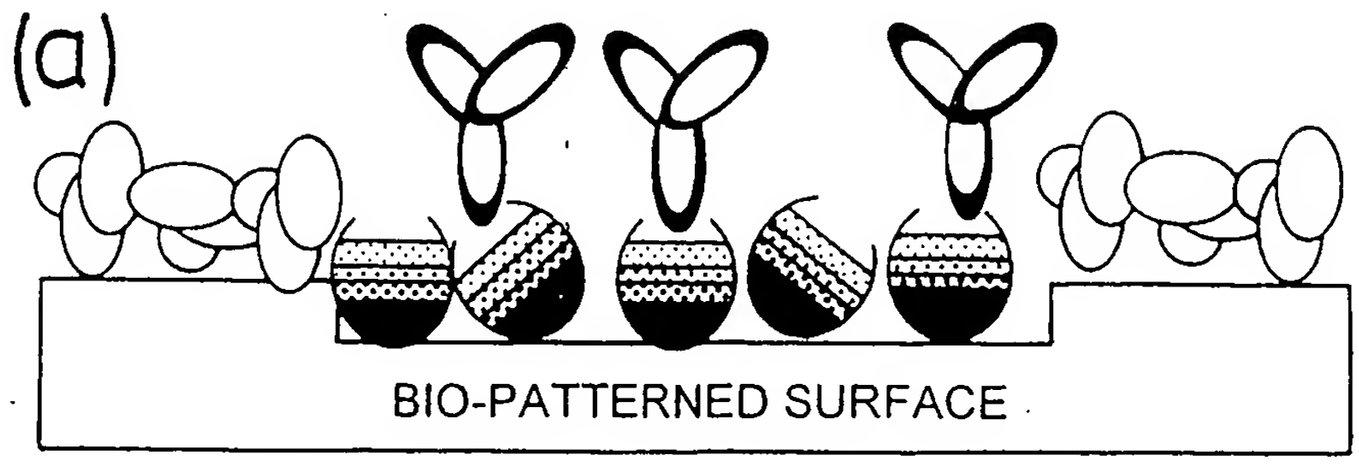

FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/03246

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropnate, of the relevant passages	Relevant to claim No.
X Y	WO 94 23295 A (ECOSSENSORS LTD ;MCALEER JEROME FRANCIS (GB); ACKLAND MARTIN ROBER) 13 October 1994 see claims see page 11, line 24 - line 28	1-20 1-20
X	WO 94 08236 A (ECOSSENSORS LTD ;TIETJE GIRAULT JORDIS (CH); SEDDON BRIAN JEFFREY) 14 April 1994 see claims see page 2, line 29 - line 33 see page 6, line 27 - page 7, line 18	1-20
Y	WO 91 08474 A (ECOSSENSORS LTD) 13 June 1991 see claims see page 3, line 7 - line 32 see page 4, line 13 - line 18	1-20

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